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# Selected Essential Amino Acid Enhancement by *Bacillus cereus* from Solid State Fermentation of Soy Pulp

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## Abstract

This research was evaluated the enhancement of essential amino acid (EAA) namely isoleucine, valine, methionine, lysine and tryptophan from soy pulp by using solid state fermentation. The EAAs were selected based on their importance and functions in fish nutrition. In this study, eight bacteria were isolated from solid state fermented soy pulp and screened for isoleucine, valine, methionine, lysine and tryptophan enhancement activities. The potential bacteria strain (S1, S2, S3, S4, S5, S6, S7, and S8) were inoculated into solid state medium (soy pulp, 95.5%; yeast extract, 2%; ammonium sulphate, 2%; 0.5% glucose v/v) and subjected to solid state fermentation for eight days. The finding of screening test of bacteria S1 assay performed the highest total EAA bacteria (42.9 ± 19.50 $gL^{-1}$). This was followed by S3 (28.2 ± 5.21 $gL^{-1}$), S7 (23.2 ± 5.29 $gL^{-1}$), S5 (18.8 ± 1.70 $gL^{-1}$), S4 (30.0 ± 14.0 $gL^{-1}$), S8 (19.9 ± 2.38 $gL^{-1}$), S2 (20.3 ± 4.31 $gL^{-1}$) and S6 (13.9 ± 0.46 $gL^{-1}$). Bacteria strain S1 was then identified as *Bacillus cereus* (MH027625) by Polymerase Chain Reaction (PCR) assay and sequencing. Therefore, the present bacteria isolate *B. cereus* (MH027625) can be used in enhancing EAA for aquaculture use.

**Keywords:** Soy pulp; Solid state; Fermentation; *Bacillus cereus;* Essential amino acids

## Introduction

Essential amino acid (EAA) is important in fish nutrition. In fish feed formulating, fish meal is the main ingredient because it contents all needed EAA. Among important EAA in fish nutrition were isoleucine, valine, methionine, lysine and tryptophan [1-3]. However, the increasing of fish meal cost become constraint in aquaculture industry development. Therefore, it is a must to find alternative EAA source. One of the rich EAA source is from plant. A recent study found alternative protein source for red hybrid tilapia using rubber seed [4]. One of the potential plants based EAA source is soy pulp. Soy pulp is a byproduct from soy milk production [5]. It can be a good potential source of low-cost plant protein. However, plant containing high anti-nutritional factor that hindering the application of plant in fish feed formulation [6]. There are several ways in minimising anti-nutrition factors such as heat processing, roasting, extrusion, and micronization [7]. Traditionally, fermentation method was used to decrease crude fibre [8]. In this study, solid state fermentation was used in minimising crude fiber in soy pulp.

The aim of present study is to reveal the potential of fermentative bacteria for EAA enhancement in soy pulp through solid state fermentation.

## Materials and Methods

### Bacteria isolation from solid state fermented soy pulp

The commercial fermented soy pulp was purchased from Prima Mekar, Malaysia. The bacteria isolations were done by using 10-fold dilution method [9] using 0.5% NaCl. The sample was vortexed for 1 min and left for five minutes to obtain supernatant. The supernatant was placed on Tryptone Soy Agar (TSA) and incubated overnight at 30°C for bacteria growth. The colonies appeared on the agar were selected morphologically and picked to on TSA for further used. The stocking of bacteria was made prior to bacteria preservation. The stock of bacteria was kept in glycerol 50% (v/v) and stored in -80°C.

### Screening of bacterial isolates for EAA production

Inoculums of isolates were prepared by inoculating the bacteria using sterile tooth pick into universal bottle containing Tryptone Soy Broth (TSB) at 30°C for 24 hrs. The bacteria screening was made by subjecting each inoculum into fermentation media. Fermentation media subjected to each isolates were prepared (soy pulp, 96%; yeast extract, 2%; ammonium sulphate, 2%; 0.5% glucose v/v). The hydrolysate was prepared individually by preparing 0.5% (v/v). The sterile mediums were inoculated isolates individually and mixed thoroughly. The flasks were incubated for 8 days at room temperature (27-30°C).

## Quantification of EAA

The sample was taken at each treatment was taken on day 0, 4, 8, and 12 of fermentation by using acidic ninhydrin method [10]. The fermentation products were collected at interval of four days to quantify tryptophan, lysine, methionine, valine and isoleucine content. About 5 g of each sample was homogenized with 10 mL of sterilized distilled water and left for 10 mins to obtain the supernatant. The supernatant was filtered using membrane with pore size of 0.45 µm. Sample preparations were done in 5 mL capped pyrex tubes. About 50 µL of supernatant from samples were mixed with 550 µL of ninhydrin reagent. The content of Pyrex tubes was heated in 100°C water bath for one hour and let to cool to room temperature. After that, 1600 µL of glacial acetic acid was added into the tubes. The data of absorbance reading of tryptophan, lysine, methionine, valine and isoleucine were taken at specific wavelength (tryptophan, 340 nm; lysine, 470 nm; methionine, 480 nm; valine, 510 nm; and isoleucine, 570 nm) [11-13] using UV Vis Spectrophotometer. The data of absorbance reading of each amino acid was converted into concentration using standard plot of the amino acid. The known

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| Variables | Tryptophan ($gL^{-1}$) | Lysine ($gL^{-1}$) | Methionine ($gL^{-1}$) | Valine ($gL^{-1}$) | Isoleucine ($gL^{-1}$) | Total ($gL^{-1}$) |
|---|---|---|---|---|---|---|
| Control | 1.9 ± 0.06 | 1.1 ± 0.17 | 7.9 ± 1.87 | 11.4 ± 2.76 | 7.3 ± 1.86 | 29.4 ± 6.70 |
| S1 | 1.9 ± 0.01 | 1.4 ± 0.52 | 11.8 ± 5.55 | 17.2 ± 8.39 | 10.6 ± 5.06 | 42.9 ± 19.50 |
| S2 | 1.8 ± 0.11 | 0.8 ± 0.13 | 5.3 ± 1.20 | 7.7 ± 1.68 | 4.8 ± 1.21 | 20.3 ± 4.31 |
| S3 | 1.9 ± 0.02 | 1.1 ± 0.16 | 7.6 ± 1.56 | 10.7 ± 2.24 | 6.9 ± 1.24 | 28.2 ± 5.21 |
| S4 | 1.5 ± 0.41 | 1.1 ± 0.34 | 8.0 ± 3.82 | 11.8 ± 5.56 | 7.7 ± 3.98 | 30.0 ± 14.0 |
| S5 | 1.8 ± 0.06 | 0.8 ± 0.06 | 4.8 ± 0.48 | 6.9 ± 0.62 | 4.5 ± 0.50 | 18.8 ± 1.70 |
| S6 | 1.6 ± 0.10 | 0.6 ± 0.02 | 3.4 ± 0.12 | 5.0 ± 0.22 | 3.2 ± 0.30 | 13.9 ± 0.46 |
| S7 | 1.9 ± 0.02 | 0.9 ± 0.15 | 6.3 ± 1.56 | 8.7 ± 2.14 | 5.5 ± 1.43 | 23.2 ± 5.29 |
| S8 | 1.7 ± 0.03 | 0.8 ± 0.07 | 5.2 ± 0.68 | 7.3 ± 0.92 | 4.8 ± 0.68 | 19.9 ± 2.38 |

**Table 1:** Mean ± S.E of 5 EAA produced by eight isolates of bacteria in screening test.

concentration of tryptophan, lysine, methionine, valine and isoleucine were prepared using same method as sample preparation to obtain standard plot of concentration versus absorbance reading.

### Statistical analysis

The result of EAA concentration collected from each isolates in this study were analysed by using analysis of one way (ANOVA), with $p<0.05$, followed by Tukey tests using statistical software package Minitab version 17.0.

### Identification of bacteria

Bacteria strain that showed the highest total EAA was identified at genetic level by Polymerase Chain Reaction (PCR) and Gram Staining. PCR reactions were performed in 25 µL PCR mixture containing 1 µL of colony DNA template, 1x PCR buffer containing 1.5 mM of $MgCl_2$, 0.25 mM of each deoxyribonucleoside triphosphate (dNTP), 0.3 µM of universal primers and 2.5 U of Taq polymerase (Bioline, UK). The primers used were forward EUB338F (5'-ACTCCTACGGGAGGCAGCAG-3') and reverse primer EUB518R (5'- ATTACCGCGGCTGCTGG -3') [14,15]. Amplifications for primers EUB338F and EUB 518R were carried out by programming the thermal cycler (Eppendorf, Germany) to the profile: 1 cycles at 95°C for 1 min; 25 cycles at 95°C for 15 s, 60°C for 15 s, and 72°C for 5 min [16]. The PCR products together with 100 bp DNA markers (Fermentas, USA) were electrophoresed on 1% agarose gel containing ethidium bromide (5 µg/µL) submerged in 1x TAE buffer.

## Results

### Screening of bacterial isolates for EAA production

Out of eight bacteria strains, S1 bacteria strain performed the highest enhancement of total EAA, stated in Table 1. Results showed the total enhancement of EAA from S1 strain bacteria was the highest (42.9 ± 19.50 $gL^{-1}$), followed by S3 (28.2 ± 5.21 $gL^{-1}$), S7 (23.2 ± 5.29 $gL^{-1}$), S5 (18.8 ± 1.70 $gL^{-1}$), S4 (30.0 ± 14.0 $gL^{-1}$), S8 (19.9 ± 2.38 $gL^{-1}$), S2 (20.3 ± 4.31 $gL^{-1}$) and S6 (13.9 ± 0.46 $gL^{-1}$). However, there were no significant differences in total production of essential amino acid for each bacteria strain isolated from solid state fermented soy pulp.

### Identification of bacteria

After PCR assay of S1strain bacteria, PCR product was sent for sequencing and resulted sequences were identified by BLAST against the National Center for Biotechnology Information's nucleotide (NCBI) database. Bacteria strain S1 showed 99% similarity to *Bacillus cereus* (MH027625).

## Discussion

In the present study, *B. cereus* (MH027625) was successfully isolated and identified from fermented soy pulp. Similar finding was also reported where two isolates of *Bacillus spp*. were successfully isolated from dochi, natural fermented food in Taiwan using nutrient agar [17]. On the other hand, same genus of bacteria, *Lactobacillus spp*. also reported using de Man-Rogosa and Sharp MRS agar [18]. Therefore, it can be said that *B. cereus* (MH027625) is one of the bacteria species that can be found in fermented food. Plus, it has been reported that *Bacillus* species are ubiquitous in nature [19]. Bacillus is well known as good protease producer [20]. This characteristic made Bacillus as a good essential amino acid enhancer in fermentation medium with high content of protein such as soy pulp. Other than bacteria, fungi and yeast should also be reported in in the fermented soy pulp. As some EAA may have better enhancement from various type of microbes, fungi and yeast should also be targeted during the isolation. Then, the microbes should be tested in the same fermentation media for EAA enhancement.

The result showed that *B. cereus* (MH027625) can enhance four out of five selected EAAs which are isoleucine, valine, methionine, and lysine but failed to enhance tryptophan. Similar finding were also reported where *Bacillus spp*. was failed to enhance tryptophan in the fermentation process [21]. Instead of enhance tryptophan; the bacteria were utilizing tryptophan in the medium. The author reported that tryptophan was utilised in some amino acid pathways such as the formation of amines in *B. cereus*. This indicates that *B. cereus* was not able to produce extra level of tryptophan. However, contra finding was reported where *Bacillus spp.* can enhance tryptophan due to presence of aromatic amino acid genes [22]. It was claimed that tryptophan utilization is involved in lysine metabolism. In addition, a study reported that tryptophan was utilized in lysine accumulation [23]. However, in the present study, tryptophan level was not decreasing but in the same level with control. This indicates that during fermentation, tryptophan was utilized in the same amount of tryptophan produced. Thus, more studies need to be carried out to verify that tryptophan to be used as lysine production.

In the present study, *B. cereus* (MH027625) successfully enhances methionine level in soy pulp fermentation. Similar finding was obtained from *Bacillus siamensis* where there was an increase in the accumulation of methionine (39.5%) as well as lysine (17.7%) [24]. In the present study, it was reported that there was a high accumulation of methionine along with lysine. However, it was reported that methionine was utilised in lysine accumulation in *Bacillus subtilis* [23]. It can be proved that different strains of Bacillus give different reaction between each EAA production. Hence some EAAs have antagonism effect with each other. Therefore, the present bacteria isolated should be focused in this area.

The present bacterial isolated was able to enhance isoleucine during fermentation process. Similar finding with higher concentration

of isoleucine compared to present study was also reported in Cho et al. [24] (26.8 $gL^{-1}$) due to its high proteolytic activity [25]. The study carried out the comparison of fermented seasoning sauces using insect with soy sauce and it was proved that protein degradation from insect is more efficient than soy protein. Also, it was reported that free essential amino acid was increased 1.5-2 times during fermentation. Therefore, it can be considered if soy pulp can be mixed with protein source from animal to have better enhancement of EAA.

## Conclusion

*Bacillus cereus* (MH027625) was successfully isolated and identified from fermented soy pulp in which can enhance lysine, methionine, valine and isoleucine but failed to enhance tryptophan. The finding of this study is important in fish feed formulating for aquaculture use.

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